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Does exposure to reduced pH and diclofenac induce oxidative stress in marine bivalves? A comparative study with the mussel *Mytilus galloprovincialis* and the clam *Ruditapes philippinarum*☆

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ABSTRACT

CO_2-driven acidification and emerging contaminants, such as pharmaceuticals, pose new threats for the maintenance of natural populations of marine organisms by interfering with their normal biochemical pathways and defences. The combined effects of seawater acidification, as predicted in climate change scenarios, and an emerging contaminant (the non-steroidal anti-inflammatory drug, NSAID, diclofenac) on oxidative stress-related parameters were investigated in the Mediterranean mussel *Mytilus galloprovincialis* and the Manila clam *Ruditapes philippinarum*. A flow-through system was used to carry out a three-week exposure experiment with the bivalves. First, the animals were exposed to only three pH values for 7 days. The pH was manipulated by dissolving CO_2 in the seawater to obtain two reduced pH treatments (pH −0.4 units and pH −0.7 units), which were compared with seawater at the natural pH level (8.1). Thereafter, the bivalves were concomitantly exposed to the three experimental pH values and environmentally relevant concentrations of diclofenac (0.00, 0.05 and 0.50 μg/L) for an additional 14 days. The activities of superoxide dismutase, catalase and cyclooxygenase, and lipid peroxidation and DNA strand-break formation were measured in both the gills and digestive gland after 7, 14 and 21 days of exposure to each experimental condition. The results show that the biochemical parameters measured in both the mussels and clams were more influenced by the reduced pH than by the contaminant or the pH*contaminant interaction, although the biomarker variation patterns differed depending on the species and tissues analysed. Generally, due to increases in its antioxidant defence, *M. galloprovincialis* was more resistant than *R. philippinarum* to both diclofenac exposure and reduced pH. Conversely, reduced pH induced a significant decrease in COX activity in both the gills and digestive gland of clams, possibly resulting in the increased DNA damage observed in the digestive gland tissue.

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1. Introduction

The continuous increase in carbon dioxide (CO_2) emissions in the atmosphere is considered one of the most important factors contributing to global climate changes (GCCs) (Solomon et al., 2009). The average atmospheric CO_2 level is currently 407 ppm (NOAA, 2017) and could reach 800 ppm by 2100 (Feely et al., 2004). Generally, atmospheric CO_2 diffuses passively into ocean surface waters, causing a reduction in pH values (acidification) (Caldeira and Wickett, 2003; Feely et al., 2009; Orr et al., 2005). Seawater pH values have already decreased by approximately 0.1 pH units with respect to pre-industrial levels, and a further reduction by 0.3–0.5 pH units is predicted before the end of the 21st century (Caldeira and Wickett, 2005; Raven et al., 2005; IPCC, 2007). Different studies have demonstrated that reduced pH may

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negatively affect marine organisms, especially species with calcareous structures (for a review, see Byrne, 2012 and Nikinma, 2013). Seawater acidification has been shown to alter the physiological parameters (Liu and He, 2012), antioxidant responses (Hu et al., 2015; Matozzo et al., 2013; Freitas et al., 2016a), immune parameters (Bibby et al., 2008; Matozzo and Marin, 2011; Matozzo et al., 2012), growth rate and survival (Chaparro et al., 2009; Gazeau et al., 2010; Byrne et al., 2013; Bressan et al., 2014; Munari et al., 2016) of marine organisms. Furthermore, biological variations in the response to seawater acidification have been reported among different species (Byrne, 2012) and between different populations of the same species (Range et al., 2013).

However, seawater acidification is not the only threat that aquatic organisms must face. In particular, species inhabiting coastal and estuarine ecosystems are continuously exposed to a wide range of anthropogenic substances, such as metals, pesticides, and pharmaceuticals and personal care products (PPCPs). In particular, PPCPs are a class of emerging environmental contaminants detected in various aquatic systems worldwide, such as seawater and freshwater surface waters, groundwater and effluents from wastewater treatment plants, in the ng/L-μg/L range (Daughton and Ternes, 1999; Kolpin et al., 2002; Metcalfe et al., 2003; Fent et al., 2006; Santos et al., 2010; Gaw et al., 2014). Even though PPCPs are generally considered to be non-persistent, their continuous release into aquatic ecosystems is a cause of concern. Indeed, PPCPs can be found in the environment either as parent compounds or as metabolites (Daughton and Ternes, 1999; Kolpin et al., 2002; Metcalfe et al., 2003; Bringolf et al., 2010). Since they are designed to be biologically active at low concentrations, there is a growing concern about their possible chronic effects on non-target aquatic species after they are released into the environment (Fent et al., 2006; Boxall et al., 2012). Among pharmaceuticals, diclofenac is one of the most commonly used non-steroidal anti-inflammatory (NSAIDs) drugs. The worldwide consumption rate of this cyclooxygenase (COX)-1 and −2 inhibitor exceeds 1000 tons per year (Zhang et al., 2008). Diclofenac is commonly used in both human and veterinary medicine (Fent et al., 2006) and can be found at concentrations ranging from ng/L to mg/L in various aquatic environments, such as wastewater treatment plant effluents and coastal regions (Buser et al., 1998; Ternes, 1998; Metcalfe et al., 2003; Ashton et al., 2004; Wiegel et al., 2004; Scheytt et al., 2005; Rodríguez-Navas et al., 2013). Despite evidence of the occurrence of NSAIDs in the aquatic environments, little is known about the possible chronic effects caused by environmentally relevant concentrations of NSAIDs on aquatic non-target species. In this regard, more studies are needed to assess the biological effects of these molecules and thereby evaluate their potential impacts on the health of natural populations. Overall, the acute and chronic toxic effects induced by NSAIDs in the form of single substances or mixtures have been moderately documented for both aquatic invertebrates and vertebrates (Parolini and Binelli, 2011; Parolini et al., 2011b; Zivna et al., 2015; Mezzelani et al., 2016). In a global climate change scenario, it is important to understand how shifts in environmental parameters, such as pH, may interact with chemical contamination. In particular, there is lack of knowledge about how the sensitivity of organisms to pollutants will be affected by the predicted future changes in environmental conditions. Due to their sedentary and filter feeding habits, marine bivalve molluscs are generally recognized as valuable bioindicators for assessing the effects of pollution and other environmental stressors on aquatic organisms (Regoli, 1998; Torres et al., 2002; Lau and Wong, 2003; Krishnakumari et al., 2006; Verlecar et al., 2006). Furthermore, they are important components of coastal and estuarine communities and are economically relevant to fisheries and farming.

The first goal of this study was to evaluate the combined effects of seawater acidification and diclofenac on two bivalve species, the Mediterranean mussel *Mytilus galloprovincialis* and the Manila clam *Ruditapes philippinarum*, with different ecological features. *M. galloprovincialis* can be found on hard substrates, and its vertical distribution is limited by its capacity to tolerate high temperatures, by desiccation in the intertidal zone during low tide events, and by the presence of hard substrates at depth. Conversely, *R. philippinarum* lives in the mid to low intertidal zone in bays, lagoons and estuaries, buried 2–4 cm below the sediment surface in mud or sand. The second goal of the study was to highlight differences in the responsiveness of the bivalve species. Adults of the two bivalve species were exposed only to three pH values for 7 days. The pH was manipulated by dissolving CO_2 in the seawater to obtain the two reduced pH treatments (pH −0.4 units and pH −0.7 units), which were compared with seawater at the natural pH level (8.1). Thereafter, the bivalves were concomitantly exposed to the three experimental pH values and environmentally relevant concentrations of diclofenac (0, 0.05 and 0.5 μg/L) for an additional 14 days. Several biomarkers indicative of oxidative stress and genotoxicity were investigated by measuring the activities of antioxidant enzymes (superoxide dismutase, SOD; catalase, CAT; cyclooxygenase, COX), lipid peroxidation (LPO) and DNA strand-break formation.

2. Materials and methods

2.1. Animals

Specimens of *M. galloprovincialis* (4.0 ± 0.5 cm shell length) and *R. philippinarum* (3.5 ± 0.5 cm shell length) were collected along the west coast of the Northern Adriatic Sea (Cavallino Treporti, Italy, temperature of approximately 25 °C, pH of 8.2) and in the Lagoon of Venice (Chioggia, Italy, temperature of approximately 15 °C, pH of 8.1), respectively, and immediately transferred to the laboratory. Bivalves were carefully checked for shell damage (damaged animals were not used for the experiments), and epibionts (such as barnacles and algae) were removed from the mussels. In the laboratory, the bivalves were maintained for a few days in aerated seawater, and the salinity, temperature and pH were adjusted to values similar to those measured at the animal collection sites. Prior to exposure, the bivalves were acclimatized to the experimental conditions by gradually increasing or decreasing the seawater temperature (2 °C per day) to 22 °C and by gradually reducing the natural pH values by about −0.4 or −0.7 units. The bivalves were fed with microalgae every 12 h (*Isochrysis galbana,* at an initial concentration of 1×10^6 cells/mL). To reduce any additional stress that might arise from spawning events during the experiments, the exposure experiments were performed when the bivalves were not sexually active (June to July for mussels and November to December for clams).

2.2. Experimental setup

The experimental flow-through system (Fig. 1) used for both the mussel and clam experiments was built inside the Hydrobiological Station 'Umberto D'Ancona' at Chioggia, Italy. It consisted of an outside sedimentation tank filled with fresh seawater (approximately 1000 L) that was pumped into two tanks (approximately 300 L each) inside the laboratory through a filtration system (from one hundred to five μm) and left to equilibrate to the required temperature. The seawater was then transferred to three tanks (approximately 120 L each), where in which the pH was adjusted to experimental values by bubbling CO_2 into the seawater with an automatic control system (ACQ110 Aquarium Controller Evolution by Aquatronica) connected to pH electrodes (ACQ310N-PH by

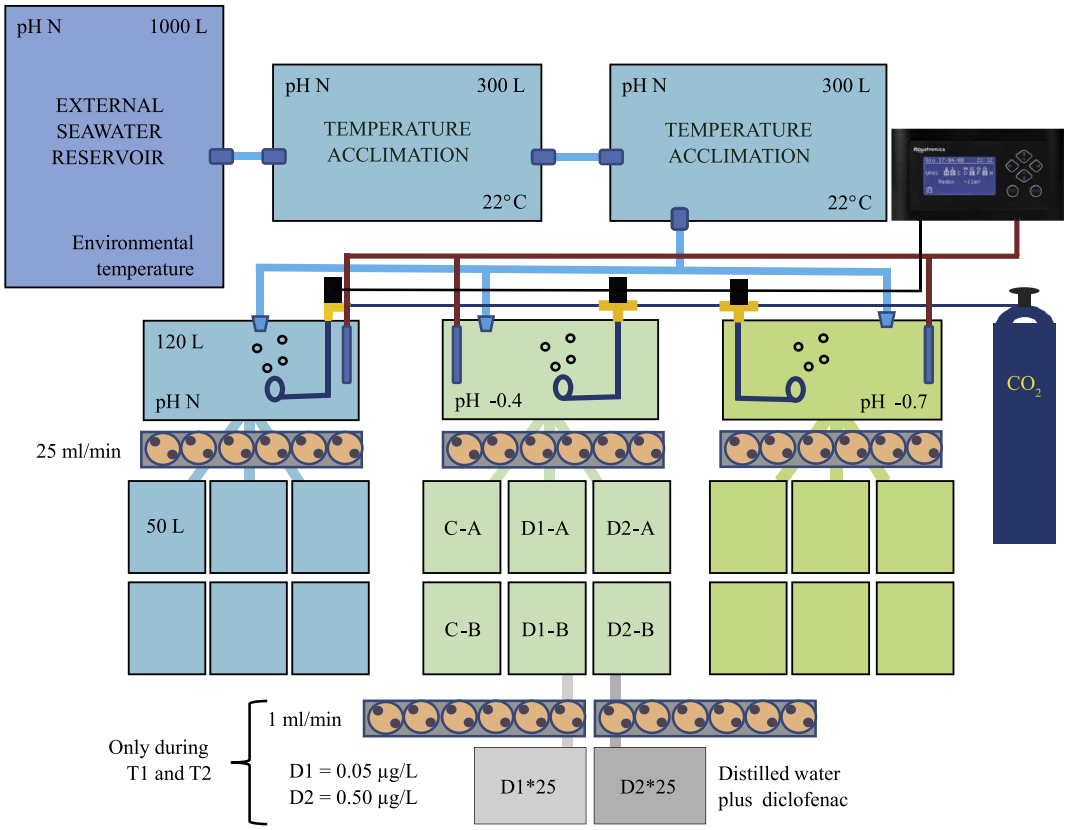

Fig. 1. The experimental flow-through system. See the M&M section for details.

Aquatronica). The seawater was finally pumped (flow speed of 25 mL/min, using ACQ450 dosing pumps by Aquatronica) into the experimental tanks (A, B and C, approximately 50 L each) containing the bivalves.

During the first week of exposure, animals were subjected to three pH conditions: the current natural pH (pH N), with a mean value of 8.07, and the mean reduced pH predicted for the year 2100 (−0.4 pH units; pH −0.4) and 2300 (−0.7 pH units; pH −0.7) (Caldeira and Wickett, 2005). During the second and third week, the animals were exposed to three concentrations of diclofenac (0.00, 0.05 and 0.50 µg/L) for each pH value. For each concentration, two replicate tanks were employed (control without contaminant: C-A, C-B; 0.05 µg/L diclofenac: D1-A, D1-B; 0.50 µg/L diclofenac: D2-A, D2-B). In total, 18 tanks (with 70 bivalves each) were prepared in the two experiments for the mussel and clam exposures.

The concentrations of diclofenac tested during the experiments were maintained at a constant level by means of a peristaltic pump with a continuous distribution of low volumes of the stock solutions (1 mL/min, using a MCP Process Pump, mod. ISM915A, provided with a MS/CA pump head, mod. ISM724A, by ISMATEC) from two 15 L glass jars to the experimental tanks. Considering the two combined flows (25 mL/min for the seawater and 1 mL/min for the contaminant solution), the concentrations of diclofenac in the two glass jars were calculated to be 25 times higher than the concentrations needed in the respective bivalve tanks. The tanks without diclofenac at pH N were used as a reference for the other treatments. During the experiments, bivalve mortality was checked daily, and animals were constantly fed by adding 250 mL of a microalgal suspension (*Isochrysis galbana*) (1×10^6 cells/mL) to each 120 L tank every 12 h, in order to maintain an algal concentration of approximately 2000 cells/mL in each experimental tank.

2.3. Tissue preparation and biomarker measurement

Tissues for the biochemical analyses were collected three times over the experiments, on day 7, after bivalves had been exposed to different pH values (T0), and on days 14 and 21, after the bivalves had been exposed to different combinations of pH values and diclofenac concentrations (T1 and T2, respectively). At each tissue sampling time, six pools (7 animals each) of gills and digestive glands were prepared for each experimental condition. The bivalve shells were opened, and the gills and the digestive gland were excised, pooled and placed in tubes on ice. Aliquots from each pooled tissue sample were immediately frozen in liquid nitrogen and stored at −80 °C until the analyses were performed. For the SOD, CAT and TBARS assays, the gills and digestive gland were thawed on ice and homogenized (1:4, w:v) in 0.1 M Tris–HCl buffer (pH 7.5) containing 0.15 M KCl, 0.5 M sucrose, 1 mM EDTA, 1 mM dithiothreitol (DTT, Sigma) and 40 µg/mL aprotinin (Sigma). They were sonicated for 2 min at 0 °C with a Braun Labsonic U sonifier at 50% duty cycles and then centrifuged at 12,000 g for 45 min at 4 °C. The supernatants (S12) were collected for further assays. For the COX assay and the DNA strand break assay, the gills and digestive gland were homogenized in ice-cold homogenization buffer (25 mM Tris-HCl, 150 mM NaCl, and 1 µg/mL Aprotinin at pH 7.5). For the COX assay, the homogenate aliquots were centrifuged at 15,000 g for 30 min at 4 °C, and the supernatant (S15) was collected. The homogenized tissues were used directly for the DNA strand break assay.

A detailed description of the methods that were used to measure the SOD (Crapo et al., 1978), CAT (Aebi, 1984) and COX activities (Gagnè et al., 2007), as well as the lipid peroxidation (Buege and Aust, 1978) and DNA strand-break formation (Gagné and Blaise,

1995), is presented in the supplementary materials (S1).

2.4. Seawater chemistry

Throughout the experiment, sea water samples were collected two to three times a week, and equal volumes taken from replicate tanks in each treatment were pooled into 250 mL polypropylene bottles. Each bottle was poisoned with 100 mL of saturated mercuric chloride ($HgCl_2$) to halt biological activity and stored at 4 °C in the dark until analysis. In total, fifty-eight seawater samples were analysed. Total alkalinity (TA) was determined via potentiometric titration by using an automatic titrator (836 Titrando, Metrohom). Each seawater sample was thermostated at 25 °C (HAAKE C25P Phoenix II, ENCO) before titration. The amount of calcium in the seawater was quantified by using an Agilent Technologies 7700× ICP-MS (Agilent Technologies International Japan, Ltd., Tokyo, Japan). The operating conditions are reported in Badocco et al. (2015). The mean concentration of calcium in the entire set of samples was 10.2 (±0.2 s.d.) mmol/L and was statistically equivalent to the 10.1 (±0.2 s.d.) mmol/L value derived from the empirical equation of Millero (1982).

The dissolved inorganic carbon (DIC) content, carbonate concentration values and CO_2 partial pressure (pCO_2) in the seawater were computed at the sampling temperature using the TA values. The application of the error propagation law allowed the computation of the standard deviations of the DIC, $CO_3^{=}$ and pCO_2 values, which were 8.4 μmol/kg, 3.4 μmol/kg and 17 μatm, respectively. All the thermodynamic equilibrium constants were computed according to Millero (1979), Millero (1995) and Millero et al. (2006). The solubility values of calcite and aragonite were obtained from Mucci (1983) and from Ingle (1975). The saturation states of calcite (Ω_{ca}) and aragonite (Ω_{ar}) were computed on the basis of the solubility products reported in Millero (1979).

2.5. Statistical analyses

For each sampling time, permutational multivariate analysis of variance (PERMANOVA, with 9999 permutations) was performed on the whole data set from each species to highlight the significant effects of pH, diclofenac and the interactions between pH and diclofenac. Subsequently, data from each biomarker in each species were analysed with PERMANOVA to determine the significant effects on the gills and digestive gland. For each biomarker, the null hypotheses tested were: at T0, no significant differences exist among pH levels (N, −0.4 and −0.7); at T1 and T2, i) for each pH tested, no significant differences exist among diclofenac concentrations (0, 0.05 and 0.5 μg L^{-1}) and ii) for each diclofenac concentration tested, no significant differences exist among pH levels (N, −0.4 and −0.7). Statistical significance was set at $p \leq 0.05$. The software package PRIMER 6 PERMANOVA Plus (PRIMER-E Ltd, Plymouth, UK) was used for the statistical analyses.

Furthermore, a canonical correlation analysis (CCA) was performed. For each sampling time (T0, T1 and T2), two sets of variables were considered in the analysis. The first set of variables included the experimental factors, namely, the pH for T0 and the pH and diclofenac concentrations for T1 and T2. Furthermore, we included the interaction term between pH and diclofenac concentration in the model. The second set of variables was defined by the measured oxidative stress-related parameters. The software package R with the CCA package (González et al., 2008) was used for the analysis.

3. Results

For both mussel and clam tissues, the biomarker results are shown in Table 1 and Figs. 2–4, whereas the PERMANOVA results for the overall data set are reported in Table 2.

3.1. M. galloprovincialis

Significant effects of pH on the overall biochemical responses of mussels were found throughout the experiment, whereas no significant effects of diclofenac were observed. A significant interaction between diclofenac and pH was recorded at T2 (Table 2). When considering the single biomarker responses, no significant effects of pH on SOD activity were observed in the mussel gills and digestive glands throughout the experiment (Tables 1 and 2). In addition, the CAT activity in gills was not significantly affected by the experimental conditions tested, whereas in the digestive gland, it was significantly influenced only by diclofenac at T1 and by the pH*diclofenac interaction at T2 (Tables 1 and 2). In particular, at T1, animals exposed to 0.5 μg/L of diclofenac showed higher CAT activity values in the digestive gland, both at pH N and −0.4, with respect to the control organisms and mussels exposed to 0.05 μg/L-diclofenac (Table 1). Conversely, at pH −0.7, no increases in CAT activity were found with increases in the diclofenac concentration. At T2, increased enzymatic activity was observed in the digestive gland of mussels maintained at pH N with 0.05 μg/L of diclofenac, at pH −0.4 without diclofenac and at pH −0.7 with 0.5 μg/L of diclofenac, when compared to mussels maintained at pH N without diclofenac (Table 1).

The COX activity was significantly influenced by pH at all sampling times in both the gills and the digestive gland, showing a U-shaped trend with decreasing pH. Conversely, no significant effects due to diclofenac or its interaction with pH were found (Fig. 2A–B; Table 2).

LPO varied slightly during the experiment and was significantly influenced only by the interaction between pH and diclofenac in the digestive gland at T2 (Tables 1 and 2).

The level of DNA damage in the gills significantly increased under reduced pH only at T2, when a significant effect of the pH*diclofenac interaction was also observed (Fig. 2C, Table 2). Conversely, DNA damage in the digestive gland was significantly influenced by pH at all sampling times. Interestingly, a reduction with decreasing pH was observed at both T0 and T1, while an increase was found at T2, when diclofenac generally increased the DNA damage (Fig. 2D, Table 2).

CCA analysis of the data obtained at T0 (corr = 0.988 and 0.947 for the first and second canonical correlations, respectively) showed a clear separation among the pH values, visually confirming the inferential PERMANOVA results (Fig. 5A). At T1 (corr = 0.999 and 0.990 for the first and second canonical correlation, respectively), a general separation was displayed among the three pH conditions, mostly between pH N and pH −0.4. In the presence of 0.5 diclofenac, pH −0.7 was associated with higher COX activity values in both the gills and digestive gland (Fig. 6A). In contrast with the observations for pH, there was no clear separation among the diclofenac concentrations, except for those at pH N. At T2 (corr = 0.999 and 0.990 for the first and second canonical correlations, respectively), the separation among the three pH conditions was more evident than that at T1 and T3, especially between pH N and the two reduced pH conditions. pH −0.7 was associated with increased COX activity in the gills and increased DNA damage in both tissues, and no clear separation among the contaminant levels was observed (Fig. 6B).

3.2. R. Philippinarum

Overall, this study demonstrated that pH significantly affected the biomarker responses in clams throughout the experiment

Table 1
SOD (U/mg protein) and CAT (U/mg protein) activities and LPO levels (nmol TBARS/mg protein) measured in bivalves. Values are the means ± SE (n = 6). T0: 7 days of exposure; T1: from day 7–14; T2: from day 14–21.

Sampling time	Conditions (pH and diclofenac)		*M. galloprovincialis*			*R. philippinarum*					
			Gills			Digestive gland			Gills			Digestive gland	
			SOD	CAT	LPO	SOD	CAT	LPO	SOD	CAT		SOD	CAT
T0	N pH		139.40 ± 8.14	25.88 ± 4.00	0.86 ± 0.04	120.85 ± 10.49	18.52 ± 2.81	1.00 ± 0.06	45.44 ± 6.47	18.90 ± 3.95		39.06 ± 5.38	15.14 ± 1.52
	pH −0.4		138.61 ± 16.02	20.09 ± 2.04	0.79 ± 0.03	188.65 ± 28.14	16.00 ± 1.53	1.08 ± 0.04	92.87 ± 31.09	18.41 ± 2.10		36.48 ± 1.90	13.95 ± 1.70
	pH −0.7		147.72 ± 7.19	21.74 ± 2.41	0.75 ± 0.04	124.93 ± 15.40	17.26 ± 1.84	1.10 ± 0.03	51.11 ± 9.38	23.77 ± 3.95		47.16 ± 5.38	15.13 ± 2.06
T1	N pH	0.00 μg/L	175.20 ± 23.56	15.14 ± 1.56	0.68 ± 0.16	172.56 ± 34.69	12.77 ± 1.57	1.05 ± 0.01	53.13 ± 3.99	27.53 ± 4.96		40.34 ± 4.02	15.97 ± 1.50
		0.05 μg/L	162.05 ± 22.65	11.50 ± 0.81	0.67 ± 0.05	161.89 ± 35.75	13.26 ± 1.05	1.01 ± 0.09	47.34 ± 1.76	22.14 ± 2.58		33.62 ± 1.73	17.19 ± 2.28
		0.50 μg/L	110.82 ± 13.15	8.74 ± 0.93	0.82 ± 0.11	115.09 ± 14.89	20.40 ± 1.66	1.07 ± 0.01	52.04 ± 2.56	22.60 ± 2.23		34.88 ± 4.04	14.84 ± 1.77
	pH −0.4	0.00 μg/L	130.72 ± 20.19	11.49 ± 1.32	0.71 ± 0.10	153.84 ± 36.55	15.32 ± 2.24	1.05 ± 0.05	60.46 ± 5.97	29.95 ± 3.79		42.01 ± 2.58	20.69 ± 3.63
		0.05 μg/L	113.75 ± 31.75	9.92 ± 2.06	0.72 ± 0.10	98.97 ± 10.17	15.65 ± 1.79	1.05 ± 0.05	52.39 ± 3.68	15.57 ± 4.44		31.29 ± 1.47	13.87 ± 1.10
		0.50 μg/L	123.66 ± 24.00	12.71 ± 1.58	0.74 ± 0.10	119.09 ± 18.64	22.74 ± 4.29	1.07 ± 0.01	50.38 ± 0.50	24.95 ± 5.40		26.80 ± 0.75	14.61 ± 3.01
	pH −0.7	0.00 μg/L	125.76 ± 15.49	12.23 ± 0.93	0.71 ± 0.12	203.74 ± 58.34	15.44 ± 1.52	1.03 ± 0.02	57.40 ± 6.47	18.59 ± 3.20		45.10 ± 6.44	16.57 ± 2.02
		0.05 μg/L	126.66 ± 9.04	14.57 ± 1.93	0.70 ± 0.09	131.66 ± 52.55	14.55 ± 1.46	1.00 ± 0.04	50.77 ± 2.11	20.37 ± 4.18		32.08 ± 1.41	12.59 ± 1.05
		0.50 μg/L	140.99 ± 12.24	10.15 ± 1.59	0.76 ± 0.09	147.27 ± 22.93	15.70 ± 1.15	1.06 ± 0.01	53.93 ± 2.27	23.07 ± 2.26		32.72 ± 1.84	16.54 ± 3.32
T2	N pH	0.00 μg/L	113.25 ± 23.34	17.44 ± 2.16	0.92 ± 0.04	153.29 ± 14.36	13.21 ± 1.20	0.99 ± 0.02	35.52 ± 1.92	25.97 ± 1.30		40.39 ± 5.27	22.67 ± 2.31
		0.05 μg/L	140.09 ± 18.74	12.56 ± 1.08	0.85 ± 0.08	181.08 ± 36.40	20.69 ± 1.69	1.00 ± 0.02	39.25 ± 0.83	27.77 ± 5.21		40.92 ± 4.21	16.92 ± 1.04
		0.50 μg/L	86.11 ± 10.58	13.34 ± 1.36	0.86 ± 0.05	130.48 ± 14.28	13.44 ± 1.43	0.97 ± 0.03	40.03 ± 1.65	15.10 ± 0.74		39.27 ± 3.18	17.08 ± 1.87
	pH −0.4	0.00 μg/L	124.98 ± 23.62	14.32 ± 0.88	0.88 ± 0.04	176.09 ± 12.41	19.60 ± 1.80	1.03 ± 0.03	45.71 ± 6.18	20.59 ± 2.27		38.45 ± 1.87	12.16 ± 0.61
		0.05 μg/L	131.41 ± 15.08	11.21 ± 1.03	0.85 ± 0.04	156.13 ± 23.75	11.80 ± 1.17	0.95 ± 0.04	42.94 ± 4.50	19.07 ± 1.78		37.57 ± 2.32	19.81 ± 5.08
		0.50 μg/L	73.04 ± 6.28	15.70 ± 1.76	0.96 ± 0.06	133.22 ± 13.39	12.48 ± 2.80	1.03 ± 0.01	45.81 ± 4.59	24.03 ± 1.81		38.54 ± 2.22	19.32 ± 2.46
	pH −0.7	0.00 μg/L	143.29 ± 20.23	16.36 ± 2.56	0.96 ± 0.02	138.82 ± 11.11	14.27 ± 1.50	0.954 ± 0.038	39.40 ± 1.80	18.28 ± 0.61		42.06 ± 4.30	15.66 ± 0.96
		0.05 μg/L	147.26 ± 32.02	14.66 ± 2.99	0.90 ± 0.07	122.25 ± 9.99	15.10 ± 1.95	0.90 ± 0.07	44.99 ± 1.11	15.27 ± 1.12		42.32 ± 3.65	19.98 ± 6.19
		0.50 μg/L	146.52 ± 17.32	13.15 ± 1.55	0.95 ± 0.07	148.37 ± 32.90	20.22 ± 1.98	0.95 ± 0.03	43.48 ± 0.85	26.00 ± 7.38		37.76 ± 1.20	21.69 ± 1.80

(Table 2). Conversely, diclofenac significantly influenced clams at T1 but not at T2, and no significant pH*diclofenac interaction was found (Table 2).

SOD activity was significantly influenced only by diclofenac at T1 in the digestive gland; in particular, at pH −0.4, a significant reduction was observed in the presence of both diclofenac concentrations (Tables 1 and 2). For both the gills and digestive gland, no significant variations in CAT activity were observed at any sampling time (Table 2).

Interestingly, pH significantly influenced COX activity in both tissues, resulting in lower activity values in clams kept at pH −0.4 and −0.7 during the experiment (Fig. 3A–B, Table 2). However, no effects of diclofenac or the pH*diclofenac interaction were recorded in the tissues.

Diclofenac significantly affected the LPO levels in the gills at T1, when an increase was observed in animals exposed to diclofenac at all pH values tested. However, in the pair-wise comparisons between the controls and diclofenac-treated clams, the effect was significant only at pH −0.4. Conversely, in the digestive gland at T1, the experimental variables and their interaction did not significantly influence LPO. At T2, pH and diclofenac significantly influenced the LPO levels in both tissues (Fig. 4A–B, Table 2).

At T0, the DNA strand-break levels showed opposite variation trends in the gills and the digestive gland with decreasing pH, although the variations were not statistically significant. Unlike the results obtained at T0, at T1, pH significantly affected both tissues, with higher levels of DNA strand-breaks under acidified conditions. Conversely, at T2, a significant effect of pH and its interaction with diclofenac was observed in the gills, with a reduction in DNA strand-break formation in clams kept under reduced pH conditions. In the digestive gland, no significant effects were found at T2 for any experimental factor (Fig. 3A–B, Table 2).

CCA analysis of the T0 results (corr = 0.988 and 0.676 for the first and second canonical correlations, respectively) showed the separation of the three pH levels, with higher COX activity at pH N (Fig. 5B). The T1 results highlighted a correlation of 0.999 for both components (Fig. 6C). Along the first component, there was a clear separation between pH N and pH −0.4 and −0.7. Furthermore, CC2 was associated with the diclofenac concentration. In particular, the control samples were clearly separated from samples treated with diclofenac at pH N and −0.4. The pH N conditions were associated with higher COX activity values in both the gills and digestive gland, while the pH −0.7 conditions were associated with an increase in DNA damage in both tissues (Fig. 6C). At T2, the pH-dependent separation defined by the first component of the CCA analysis persisted (corr = 0.999) and became more evident, as observed for pH N and pH −0.4 and −0.7. The pH N conditions were associated with high COX activity levels in both tissues (Fig. 6D). The second component (corr = 0.990) highlighted a diclofenac-dependent effect, with a clear separation between the controls and treatments that was more evident at the lowest pH. A correlation between increases in LPO and decreases in pH was observed in both tissues (Fig. 6D).

3.3. Water chemistry

Seawater carbonate chemistry data are reported in Table 3. In the experiments with mussels and clams, DIC and pCO_2 increased with decreasing pH at each sampling time. Similarly, reductions in the calcite and the aragonite saturation states were observed with decreasing pH, although they did not drop below the saturation level (<1). Only slight and erratic variations in the carbonate chemistry parameters were observed in the presence of diclofenac with respect to the control conditions.

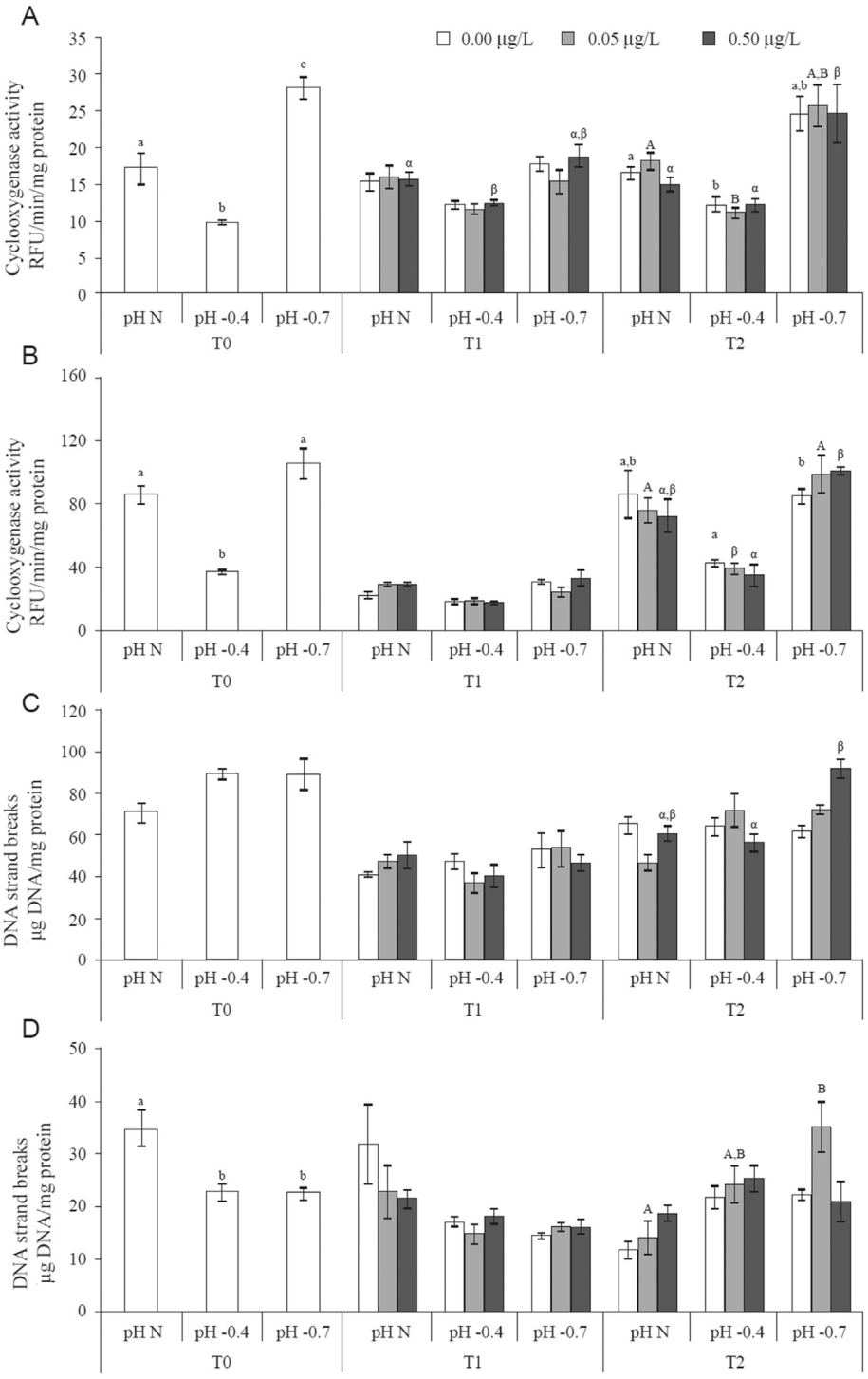

Fig. 2. COX activity (A–B) and DNA strand break levels (C–D) in the gills (A–C) and digestive gland (B–D) of *M. galloprovincialis*. Values are the means ± SE (n = 6). Significant differences ($p < 0.05$) among pH N, pH −0.4 and pH −0.7 are presented with lower-case letters (a–c) at the diclofenac concentration of 0.00 µg/L, with capital letters (A–C) at the diclofenac concentration 0.05 µg/L and with Greek letters (α-γ) at the diclofenac concentration of 0.50 µg/L. At each pH value, asterisks represent significant biomarker variations among different diclofenac concentrations.

4. Discussion

Marine organisms are simultaneously exposed to a wide range of different environmental and anthropogenic stressors in their natural environments. Although there are several scientific studies about how climate changes may influence the fate and behaviour of environmental pollutants (MacDonald et al., 2005; Wrona et al., 2005; Schiedek et al., 2007; Götze et al., 2014), the interactive effects of these stressors on marine organisms have only recently been considered (Sokolova and Lannig, 2008; Delorenzo, 2015; Gamain et al., 2016; Munari et al., 2016). In particular, there is very limited knowledge about the combined impacts of CO_2-driven acidification and emerging contaminants, such as pharmaceuticals (Freitas et al., 2016a). To fill this gap, in this study, the combined

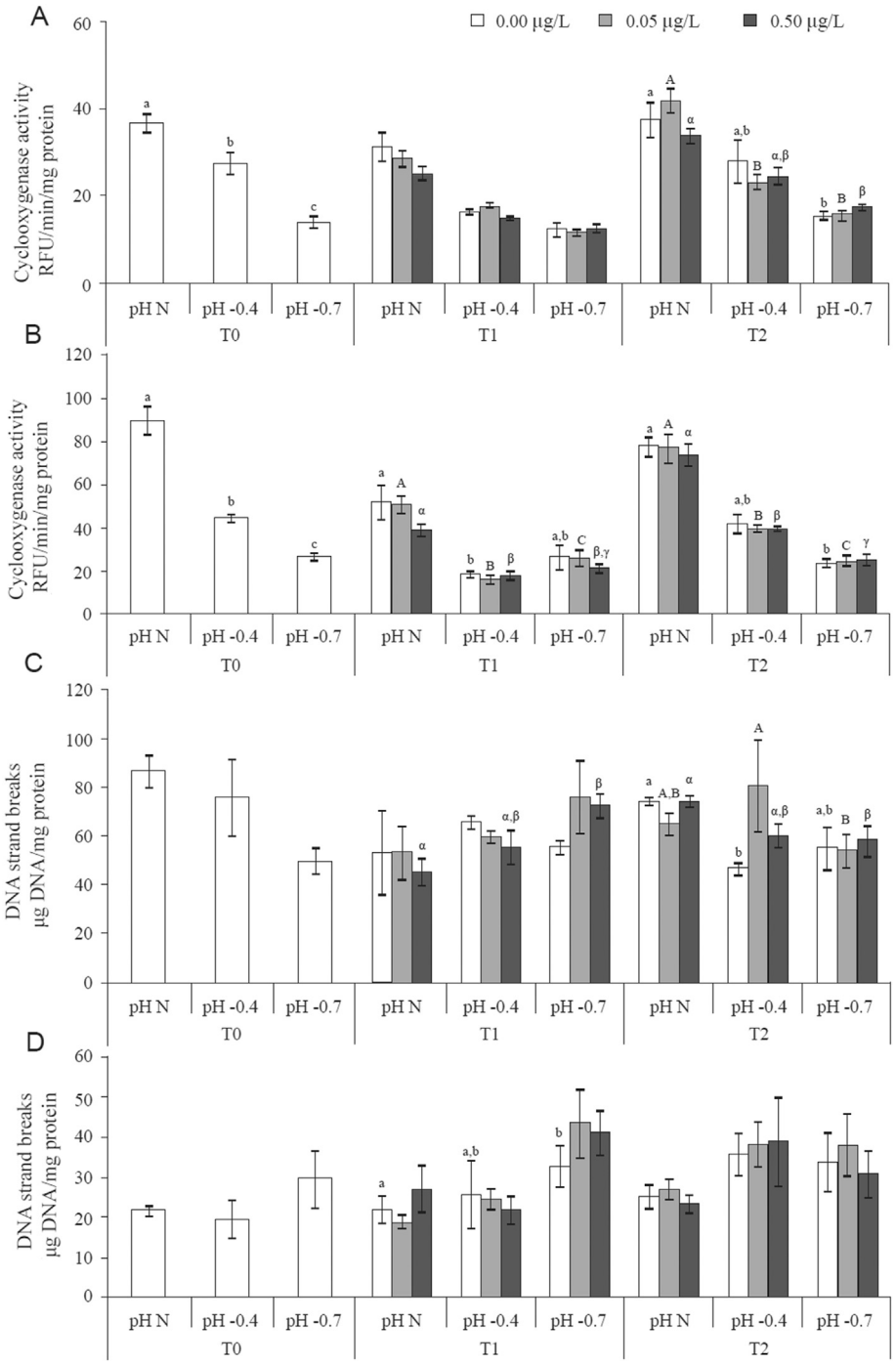

Fig. 3. COX activity (A–B) and DNA strand break levels (C–D) in the gills (A–C) and digestive gland (B–D) of *R. philippinarum*. Values are the means ± SE (n = 6). Significant differences ($p < 0.05$) among pH N, pH −0.4 and pH −0.7 are presented with lower-case letters (a–c) at the diclofenac concentration of 0.00 µg/L, with capital letters (A–C) at the diclofenac concentration 0.05 µg/L and with Greek letters (α-γ) at the diclofenac concentration of 0.50 µg/L. At each pH value, asterisks represent significant biomarker variations among different diclofenac concentrations.

effects of reduced pH and diclofenac on oxidative stress-related parameters were evaluated in *M. galloprovincialis* and *R. philippinarum* for the first time.

Both bivalve species are often used to monitor marine coastal pollution and to understand the potential effects of various pollutants in laboratory experiments (Blasco and Puppo, 1999; Ji et al., 2006; Matozzo et al., 2010; Milan et al., 2013; Munari et al., 2014; Marisa et al., 2016). Environmental and anthropogenic stressors can lead to different alterations, such as oxidative stress, at both cellular and organism levels in marine bivalves (Livingstone, 2001; Lushchak, 2011). Oxidative stress occurs when antioxidant enzymes (such as SOD, CAT and COX) are overwhelmed by the reactive oxygen species (ROS) produced in organism tissues (Soldatov et al., 2007). In bivalves, increased ROS levels may result in either the induction or inhibition of antioxidant enzymes. The reduction in antioxidant defences may result in damage to the cell membrane, lipids, DNA and proteins. The measurement of antioxidant enzyme activities and the evaluation of potential damages can be helpful to

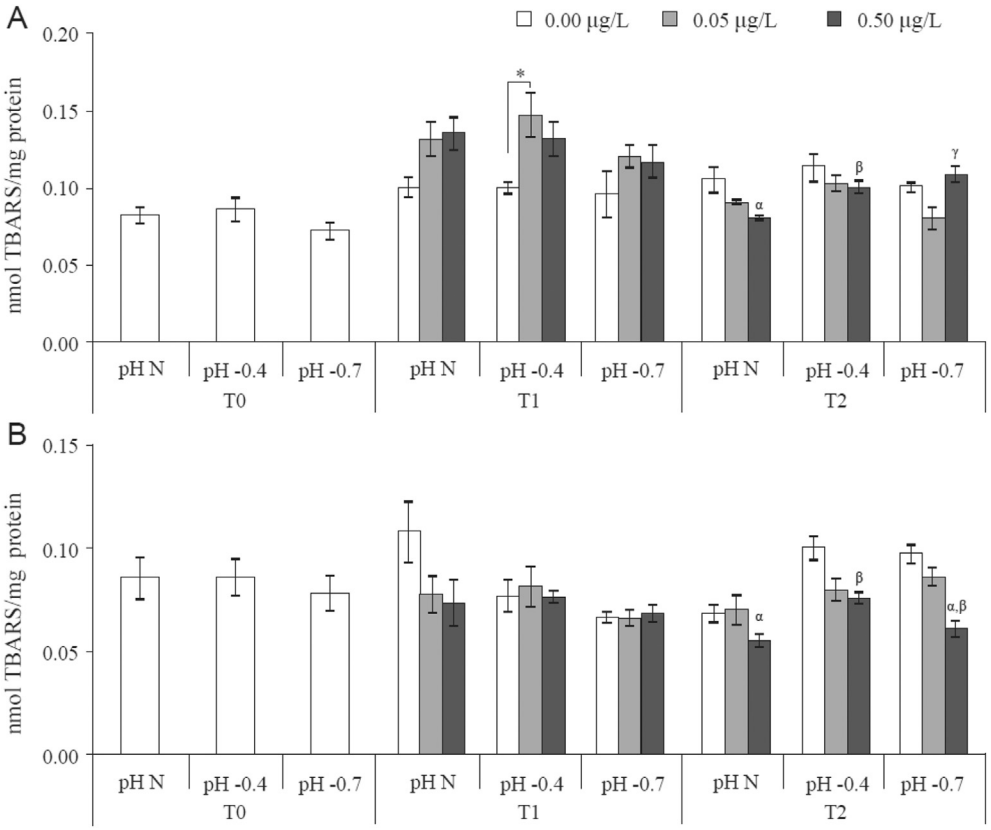

Fig. 4. LPO levels in the gills (A) and digestive gland (B) of *R. philippinarum*. Values are the means ± SE (n = 6). Significant differences ($p < 0.05$) among pH N, pH −0.4 and pH −0.7 are presented with lower-case letters (a–c) at the diclofenac concentration of 0.00 µg/L, with capital letters (A–C) at the diclofenac concentration 0.05 µg/L and with Greek letters (α-γ) at the diclofenac concentration of 0.50 µg/L. At each pH value, asterisks represent significant biomarker variations among different diclofenac concentrations.

Table 2
PERMANOVA results. The p values for all the biomarkers measured in gills and digestive gland from bivalves during the experiments carried out at three pH levels (pH N, pH −0.4 and pH −0.7), in the absence (T0: 7-days exposure) or in the presence of 0.05 and 0.50 µg/L diclofenac (T1: from day 7–14; T2: from day 14–21) are reported. Significant results are in bold.

Species	Sampling time	Factors	All data set in	Gills					Digestive gland				
				SOD	CAT	COX	DNA s.b.	LPO	SOD	CAT	COX	DNA s.b.	LPO
M. galloprovincialis	T0	pH	**0.001**	0.889	0.511	**0.000**	0.190	0.055	0.080	0.715	**0.000**	0.230	**0.003**
	T1	pH	**0.022**	0.179	0.153	**0.000**	0.999	0.254	0.549	0.125	**0.004**	0.731	**0.028**
		diclofenac	0.126	0.387	0.133	0.330	0.099	0.960	0.317	**0.005**	0.407	0.457	0.609
		pH*diclofenac	0.487	0.169	0.180	0.480	0.558	0.584	0.901	0.168	0.246	0.978	0.538
	T2	pH	**0.001**	0.589	0.735	**0.000**	0.867	**0.007**	0.676	0.092	**0.000**	0.210	**0.001**
		diclofenac	0.151	0.218	0.171	0.719	0.343	0.279	0.673	0.460	0.964	0.817	**0.041**
		pH*diclofenac	**0.014**	0.120	0.074	0.780	0.793	**0.007**	0.676	**0.001**	0.561	**0.040**	**0.025**
R. philippinarum	T0	pH	**0.003**	0.195	0.492	**0.000**	0.320	0.059	0.251	0.864	**0.000**	0.818	0.351
	T1	pH	**0.001**	0.589	0.516	**0.003**	0.161	**0.012**	0.553	0.868	**0.000**	0.080	**0.002**
		diclofenac	**0.018**	0.208	0.189	0.677	**0.003**	0.487	**0.012**	0.359	0.124	0.321	0.674
		pH*diclofenac	0.497	0.865	0.308	0.434	0.710	0.151	0.740	0.530	0.380	0.253	0.433
	T2	pH	**0.003**	0.103	0.877	**0.000**	**0.025**	**0.018**	0.718	0.801	**0.000**	**0.006**	0.173
		diclofenac	0.305	0.333	0.799	0.648	**0.026**	0.234	0.757	0.676	0.897	**0.004**	0.843
		pH*diclofenac	0.478	0.903	0.426	0.273	**0.041**	**0.021**	0.923	0.388	0.966	0.278	0.986

reveal oxidative stress induced by deleterious conditions, such as seawater acidification and the exposure to environmental contaminants. In this regard, comparisons between our results and data from the literature are difficult because, to our knowledge, no previous studies have investigated the combined effects of reduced pH and diclofenac, or other NSAIDs, on bivalve antioxidant responses. Instead, there is a considerable amount of information about the effects of single (Tomanek et al., 2011; Zhang et al., 2012; Gonzalez-Rey and Bebianno, 2014; Freitas et al., 2016b) and, to a lesser extent, combined environmental stressors (Matoo et al., 2013; Matozzo et al., 2013; Munari et al., 2016; Freitas et al., 2016a, 2016c) on antioxidant defences in aquatic invertebrates.

Among antioxidants, SOD catalyses the dismutation of superoxide anion to hydrogen peroxide in mitochondria and cytosol, and generally, an increase in this enzyme indicates the ability to cope with a possible oxidative stress. In this study, a general reduction in SOD activity was observed at T1 in both the gills and digestive gland of bivalves exposed to diclofenac at pH −0.7, even though this reduction was significant only in the clam digestive gland. The trend recorded at T1 did not persist until T2 in clams, while in

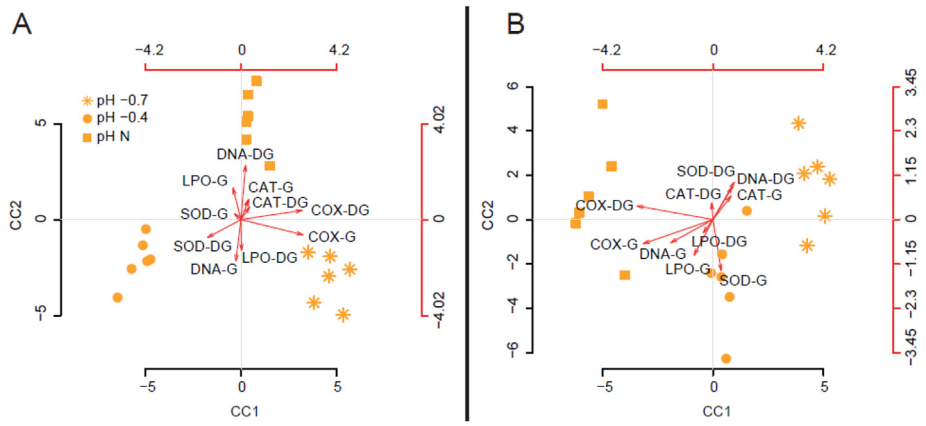

Fig. 5. Canonical correlation analysis (CCA) results for the biochemical responses in *M. galloprovincialis* (A) and *R. philippinarum* (B) exposed to different pH levels (pH N, pH −0.4 and pH −0.7) at T0. CAT: catalase, SOD: superoxide dismutase, COX: cyclooxygenase, LPO: lipid peroxidation, DNA: DNA strand breaks; -G: gills, -DG: digestive gland.

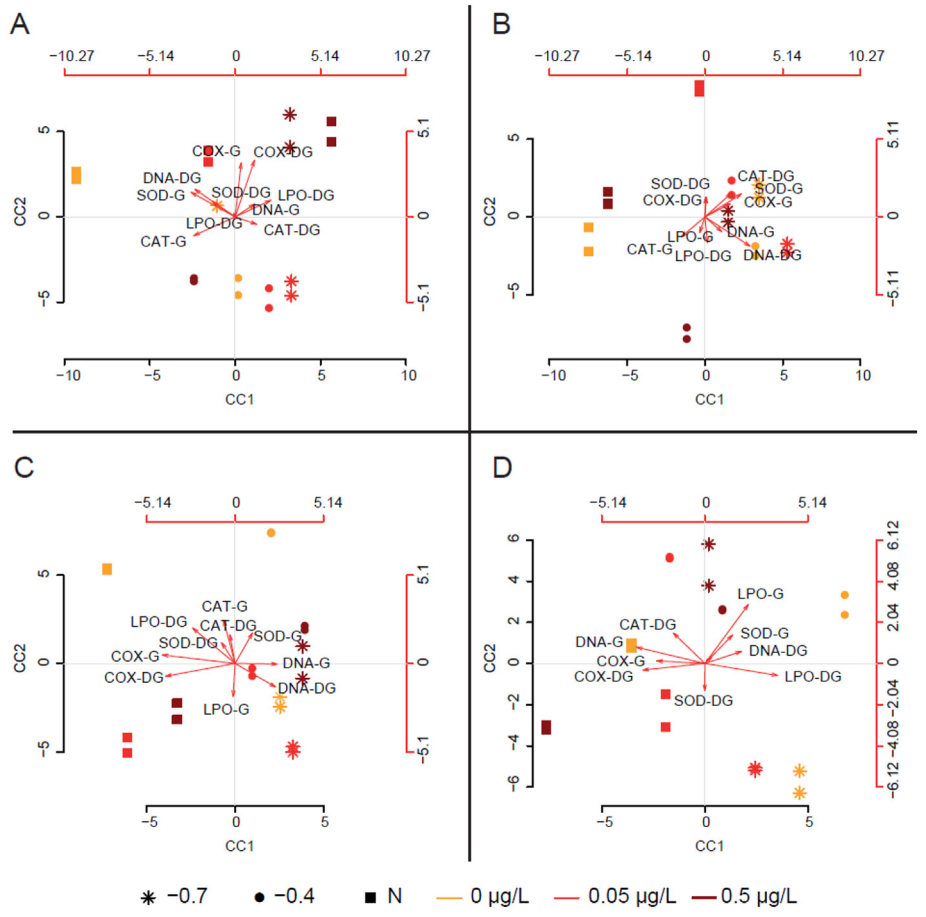

Fig. 6. Canonical correlation analysis (CCA) results for biochemical responses in *M. galloprovincialis* (A–B) and *R. philippinarum* (C–D) exposed to different pH levels (pH N, pH −0.4 and pH −0.7) and diclofenac concentrations (0.00 µg/L, 0.05 µg/L and 0.50 µg/L) at T1 (A–C) and T2 (B–D). CAT: catalase, SOD: superoxide dismutase, COX: cyclooxygenase, LPO: lipid peroxidation, DNA: DNA strand breaks; -G: gills, -DG: digestive gland.

mussels, a further decrease in SOD activity was found after the exposure to 0.5 µg/L of diclofenac at pH N and pH −0.4. Based on these findings, it is possible to infer that the contaminant generally decreased SOD activity after one week of exposure, while after two weeks, only clams seemed capable to restore control-level activity independent of treatment. Similarly, Parolini et al. (2011) reported negligible effects on SOD activity in *Dreissena polymorpha* exposed to environmental concentrations of diclofenac (0.3, 1, and 2 nM) for 96 h. In another study on *M. galloprovincialis* exposed to 250 ng/L of diclofenac, SOD activity increased significantly after 3 and 7 days in the gills and digestive gland, respectively, but it decreased in both tissues after 15 days (Gonzalez-Rey and Bebianno, 2014).

In this study, no significant changes in CAT activity were found in the bivalve tissues, except for the mussel digestive gland at T1.

Table 3
Seawater carbonate chemistry variables (mean values ± SE) recorded during the experiment. TA = total alkalinity; DIC = total dissolved inorganic carbon; pCO2 = CO2 partial pressure; Ωca = calcite saturation state; Ωar = aragonite saturation state.

Species	Sampling time	Conditions (pH and diclofenac)		pH_T	TA	DIC	pCO_2	Ω_{cal}	Ω_{arg}
M. galloprovincialis	T0	N pH		7.98 ± 0.01	2884.42 ± 52.45	2665.50 ± 50.58	631.47 ± 22.02	5.718 ± 0.11	3.76 ± 0.07
		pH −0.4		7.76 ± 0.01	2800.33 ± 12.39	2681.52 ± 8.95	1080.65 ± 20.59	3.62 ± 0.08	2.38 ± 0.05
		pH −0.7		7.34 ± 0.05	2894.53 ± 40.94	2923.77 ± 32.32	3248.09 ± 376.73	1.56 ± 0.17	1.02 ± 0.11
	T1-T2	N pH	0.00 μg/L	8.14 ± 0.01	2848.91 ± 5.40	2539.97 ± 4.86	399.19 ± 8.67	7.71 ± 0.15	5.07 ± 0.10
			0.05 μg/L	8.14 ± 0.01	2840.80 ± 2.45	2529.65 ± 7.58	391.60 ± 10.54	7.76 ± 0.17	5.10 ± 0.11
			0.50 μg/L	8.14 ± 0.01	2841.81 ± 6.30	2531.97 ± 5.32	395.75 ± 5.85	7.73 ± 0.09	5.09 ± 0.06
		pH −0.4	0.00 μg/L	7.71 ± 0.03	2837.30 ± 7.66	2734.99 ± 17.47	1262.87 ± 113.96	3.34 ± 0.24	2.20 ± 0.16
			0.05 μg/L	7.73 ± 0.02	2825.60 ± 5.93	2713.87 ± 8.83	1167.10 ± 62.20	3.49 ± 0.14	2.30 ± 0.09
			0.50 μg/L	7.74 ± 0.02	2820.23 ± 6.24	2708.33 ± 10.45	1143.96 ± 47.08	3.49 ± 0.10	2.29 ± 0.07
		pH −0.7	0.00 μg/L	7.39 ± 0.02	2879.65 ± 11.71	2886.92 ± 15.69	2734.47 ± 106.67	1.72 ± 0.07	1.13 ± 0.04
			0.05 μg/L	7.42 ± 0.01	2883.54 ± 12.98	2882.98 ± 15.51	2566.18 ± 85.56	1.81 ± 0.06	1.19 ± 0.04
			0.50 μg/L	7.43 ± 0.01	2878.18 ± 9.64	2872.24 ± 12.93	2504.41 ± 81.93	1.87 ± 0.06	1.23 ± 0.04
R. philippinarum	T0	N pH		8.05 ± 0.01	2858.26 ± 11.70	2619.42 ± 10.58	507.69 ± 6.75	6.284 ± 0.07	4.11 ± 0.05
		pH −0.4		7.67 ± 0.01	2902.09 ± 29.73	2824.05 ± 25.73	1381.66 ± 41.84	2.97 ± 0.11	1.94 ± 0.07
		pH −0.7		7.40 ± 0.01	2943.55 ± 20.34	2961.07 ± 18.40	2754.65 ± 52.17	1.67 ± 0.04	1.09 ± 0.03
	T1-T2	N pH	0.00 μg/L	8.04 ± 0.04	2802.86 ± 15.36	2604.32 ± 19.68	619.54 ± 69.33	5.56 ± 0.53	3.62 ± 0.34
			0.05 μg/L	8.05 ± 0.06	2813.08 ± 0.01	2607.14 ± 19.83	598.75 ± 62.38	5.73 ± 0.59	3.74 ± 0.39
			0.50 μg/L	8.03 ± 0.07	2803.07 ± 9.76	2597.23 ± 25.03	589.34 ± 57.84	5.72 ± 0.54	3.73 ± 0.35
		pH −0.4	0.00 μg/L	7.70 ± 0.05	2790.57 ± 13.17	2708.01 ± 21.89	1267.68 ± 131.59	3.18 ± 0.27	2.07 ± 0.18
			0.05 μg/L	7.67 ± 0.03	2784.87 ± 15.65	2712.67 ± 18.96	1351.03 ± 112.03	2.94 ± 0.23	1.92 ± 0.15
			0.50 μg/L	7.63 ± 0.06	2795.43 ± 13.05	2737.57 ± 28.50	1557.71 ± 257.96	2.70 ± 0.28	1.76 ± 0.18
		pH −0.7	0.00 μg/L	7.37 ± 0.01	2799.51 ± 24.39	2813.39 ± 24.13	2573.12 ± 206.00	1.69 ± 0.17	1.10 ± 0.11
			0.05 μg/L	7.41 ± 0.03	2824.33 ± 25.75	2824.98 ± 37.76	2364.37 ± 238.99	1.87 ± 0.17	1.22 ± 0.11
			0.50 μg/L	7.36 ± 0.02	2834.92 ± 22.72	2853.06 ± 35.96	2696.15 ± 281.88	1.67 ± 0.18	1.09 ± 0.12

Indeed, at T1, remarkable increases in CAT activity were recorded in mussels exposed to 0.5 μg/L of diclofenac at pH N and pH −0.4. Conversely, Gonzalez-Rey and Bebianno (2011) did not find any significant variations in CAT activity in the gills and digestive gland of *M. galloprovincialis* exposed to 0.25 μg/L of diclofenac. Based on the available data, the exposure to other NSAIDs can induce CAT activity. Parolini and Binelli (2011) demonstrated that the exposure of *D. polymorpha* to 0.2 μg/L of ibuprofen for 4 days significantly induced CAT activity in soft tissues. Similarly, in the amphipod *Hyalella azteca*, a 72-h exposure period to diclofenac-contaminated sediment significantly increased CAT activity (Oviedo-Gómez et al., 2010).

The activity of antioxidant enzymes can also be influenced by variations in environmental parameters (e.g., temperature, pH and salinity values); seasonal changes in endogenous factors (e.g., gonad development, reproduction and metabolic requirements) may amplify the effects of GCCs on antioxidant defences (Bocchetti and Regoli, 2006), even though the duration of the exposure to a certain stressor plays an important role in the antioxidant response. In the copepod *Centropages tenuiremis*, exposure to elevated pCO_2 conditions caused a significant decrease in SOD activity after 1 day, with enzyme activity increasing on day 2 and decreasing on days 3 and 4 (Zhang et al., 2012). In another study that exposed *M. galloprovincialis* and *C. gallina* to three different pH levels (8.1, 7.7 and 7.4) combined with two temperatures (22 and 28 °C), Matozzo et al. (2013) found that CAT activity increased under low pH and high temperature conditions, mostly in the clam digestive gland. Overall, in both the present and that by Matozzo et al. (2013), no evident relationship between SOD activity and CAT activity has been observed.

The measurement of cyclooxygenase (COX) activity is generally considered a biomarker of oxidative stress, even if COX can be involved in other important biological processes, such as immune responses, gonad maturation and spawning (Gagnè et al., 2007). In both the mussels and clams, the COX activity measured in the gills and digestive gland at all sampling times was significantly influenced only by pH, with different response patterns between the two species. In both mussel tissues, the lowest COX levels were found in animals kept at pH −0.4, and the highest levels were found in animals kept at pH-0.7. However, in clam tissues, the lowest COX activity was always observed at pH −0.7. This is the first report on the effects of seawater acidification on COX activity. Reduced pH may have altered COX functionality or its expression. Further studies are needed to understand the role of shifts in environmental parameters in modulating this enzyme. Unlike the results of other studies, in our experiments, COX was not influenced by diclofenac at the concentrations tested. In the freshwater mussel *Elliptio complanata*, a two-week exposure period to domestic wastewater aeration lagoons in the eastern shores of the Richelieu River (Quebec, Canada), significantly increased COX activity, probably due to the presence of several pharmaceuticals (Gagnè et al., 2007). However, the concentrations of pharmaceuticals, including diclofenac, in riverine waters receiving effluents from wastewater treatment plants are known to be even ten times higher than those in the surface waters of estuarine and coastal environments (Rodríguez-Navas et al., 2013), which are in the concentration range used in this study.

LPO is a well-established mechanism of cellular injury, and its measurement is used as an indicator of oxidative damage in cells and tissues. In this study, both in the mussel gills and digestive gland, no significant increases in LPO were recorded, and, over the entire experiment, the LPO values were rather stable for all conditions tested. Conversely, in clams, significant variations were observed in both the gills and the digestive gland, but the tissues demonstrated different trends. In gills, increased LPO values were observed at T1 in animals exposed to diclofenac at all pH values tested, whereas a decrease in LPO occurred at T2, except for the highest concentration of diclofenac at the lowest pH. In the clam digestive gland, at T1, the LPO levels under all the experimental conditions tested were lower than that of the control, while at T2, significant increases in LPO were observed in the animals exposed to the reduced pH values. At the same time, the animals exposed to diclofenac showed lower LPO levels than the respective control animals. We suggest that short-term exposure (T1) affected mostly the gills, which are directly exposed to the environment, whereas a longer exposure (T2) resulted in cellular membrane damage in

more internal tissues, such as the digestive gland. Overall, considering that no significant variations in LPO levels were observed in mussels and that a different pattern of variation was recorded in the two clam tissues, no clear relationship between LPO and antioxidant defences can be postulated in this study.

DNA strand-break formation was used in this study as an index of oxidative damage. The mussel gills were significantly affected by the experimental conditions by the first week of exposure, when DNA strand-break formation increased in mussels exposed to low pH. At the end of the experiment, the interaction between low pH and diclofenac resulted in the highest levels of DNA strand breaks. Significant variations in DNA damage were also observed in the mussel digestive gland at T0, T1 and T2. Unlike in the gills, however, a decreased genotoxic effect was found at low pH levels after the first week of exposure. Interestingly, in mussels maintained at pH −0.7, both the increase in DNA strand-break formation observed in the gills and the decrease in DNA strand-break formation observed in the digestive gland corresponded to an increase in COX activity in both tissues. These results suggest that, under reduced pH conditions, mussels cope with increased oxidative stress, even if, in gills, this response was not able to prevent genotoxic effects, probably because the gills directly experience seawater changes. In addition to the CAT and SOD activities, it is possible that other antioxidant defences that are well known in marine bivalves (Matozzo et al., 2013; Marisa et al., 2016), such as glutathione, were activated by the experimental conditions, thus reducing potential oxidative damage.

Unlike the mussels, the clams did not show significant variations in genotoxicity after one week of exposure to low pH. At T1, increased DNA strand-break formation was observed in both tissues of clams exposed to pH-0.7 for all diclofenac concentrations. The same pattern was maintained in the digestive gland at T2, whereas a reverse behaviour occurred in the gills, which showed a significant reduction in DNA damage. At the end of the experiment, other mechanisms of DNA protection and repair may have led to decreased DNA damage in the gills of clams kept at reduced pH. In a previous study, Tomanek et al. (2011) suggested that elevated CO_2 levels may cause oxidative stress by increasing the production of ROS, either indirectly by lowering intra- and extracellular pH, which may enhance the Fenton reaction, or directly by interacting with other ROS to form more free radicals. Although the SOD and CAT activities generally did not show significant changes under the different experimental conditions in our study, we must note that other enzymatic and non-enzymatic ROS scavengers that were not measured in this study may have played a relevant role in antioxidant defence. Although ROS level measurements were not performed, the increase in ROS under low pH can be inferred by the increased oxidative damage, resulting in higher LPO for clams and higher DNA strand-break formation for mussels.

Overall, both species considered in this study were influenced by reduced pH more than they were influenced by diclofenac, even though interactions between the two variables were often highlighted. Intertidal and shallow water species that routinely experience fluctuations in temperature, salinity and pH, species that naturally experience water with low pH due to upwelling, and infaunal species inhabiting low pH sediments may have some resilience to warming and/or acidification (Talmage and Gobler, 2009, 2010; Matson et al., 2012; Wolfe et al., 2013). In contrast, species from relatively invariable habitats may be more vulnerable to warming and/or acidification (Melzner et al., 2011). Although the two species used in this study are both intertidal and have proven to be relatively tolerant to environmental stressors, they are representative of different habitats with different characteristics. The mussel species lives in the water column attached to hard substrates, and the clam species burrows in sediments. Considering the ecological features of the studied species, the mussels could be expected to be more tolerant to shifts in environmental parameters. Indeed, a higher capability to modulate biochemical and immune-related responses to cope with environmental stresses, such as increased temperature and reduced pH, was revealed in *M. galloprovincialis* compared with an another venerid clam, *Chamelea gallina* (Matozzo et al., 2012, 2013). In addition, *M. galloprovincialis* was even found to be more resistant to aerial exposure and thermal stress than its sibling species *Mytilus trossulus* (Schneider, 2008). An additional factor in the lower resilience of *R. philippinarum* than *M. galloprovincialis* to reduced pH could be because of the extensive use of the clam in aquaculture worldwide, particularly in the Northern Adriatic lagoons. Aquaculture practices, such as the production of offspring in hatcheries from few parents, represent a potential risk for reduced genetic variability and thus may increase susceptibility to environmental challenges, such as diseases, changes in environmental parameters and pollution (Mura et al., 2012; Xing et al., 2014).

5. Conclusion

In conclusion, comparing the responsiveness of the two species selected as model organisms in this study, *R. philippinarum* appears to be more sensitive than *M. galloprovincialis* to predicted reductions in pH. In particular, it is important to highlight the opposite pattern of COX activity variations in *R. philippinarum* and *M. galloprovincialis*. While COX activity increased in the mussels exposed to low pH, a decrease in COX activity was found in the clams. COX inhibition results in the decreased biosynthesis of prostaglandins, which are involved in many physiological processes, such as reproduction, water transport and osmoregulation (Ruggeri and Thoroughgood, 1985; Osada and Nomura, 1990). Considering that prostaglandins are known to be implicated in gonad maturation and spawning in scallops (Osada and Nomura, 1990), the reduction in COX activity observed in clams is a cause for concern and requires in-depth investigation in future studies. Understanding the role of COX and the potential interference of environmental stressors, such as seawater acidification and emerging contaminants, on the reproduction, growth and survival of marine organisms is crucial for the maintenance of natural populations and the preservation of ecological health in marine environments.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.envpol.2018.05.005.

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